application documents

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Polyethylene glycol-derivatized biomolecules and their use in heterogeneous detection methods

Polyethylene glycol-derivatized ~~biomolecules and their use in heterogeneous detection methods~~

Description

The invention concerns methods for the detection of an analyte in a sample and suitable reagent kits for such methods.

Methods for the detection of an analyte in a sample in which a solid phase is used are called heterogeneous test formats. A problem in such methods is that components of the sample or of the test reagents often bind unspecifically to the solid phase which can lead to false test results. This unspecific binding is observed particularly frequently in samples from body fluids e.g. serum or plasma. In order to suppress this unspecific binding it is possible to add surface active substances, such as e.g. Tween 20, to the reagents (WO 88/07683). In addition it is known that metallic surfaces (Whitesides et al., Science 252 (1991), 1164-1166) and oxidic surfaces (EP-A-0 664 452) can be functionalized with reactive polyethylene glycol derivatives in order to minimize unspecific binding on the surface.

The surface active substances of the prior art used to reduce unspecific binding have the disadvantage that they displace molecules, such as solid phase receptors, bound to the solid phase and in this manner impair the test function. Furthermore, detergent surfactants produced on an industrial scale which have a heterogeneous composition and occasionally contain impurities are usually used as surface active

substances. The resulting batch variations often lead to interferences and non-reproducible results. Moreover the structure of sensitive solid phase molecules such as proteins may be disturbed by the surface active substances and they may be ultimately denatured.

The functionalization of metallic or oxidic surfaces with polyethylene glycol known from the prior art is, on the one hand, limited to certain types of surfaces and, on the other hand, is not sufficient to prevent unspecific binding to a layer of biomolecules applied to a solid phase surface.

Hence the object of the present invention was to provide a new method for reducing unspecific binding to a solid phase when an analyte is detected in a sample in which it is possible to at least partially avoid the disadvantages of the prior art.

A first aspect of the present invention concerns a method for the detection of an analyte in a sample comprising the steps:

(a) preparing a solid phase comprising, in an immobilized form, an analyte-specific solid phase reactant and an analyte-unspecific biomolecule which is coupled to a poly(C_2-C_3)-alkylene oxide,

(b) incubating the sample with the solid phase and a test reagent and

(c) detecting the presence or/and the amount of the analyte in the sample.

Blocking the solid phase with a polyalkylene oxide, in particular with a polyethylene glycol-modified analyte-unspecific biomolecule, resulted in a considerable

reduction of the unspecific binding of sample components to the solid phase without simultaneously significantly impairing the test sensitivity. The blocking reagent can be added during or/and after immobilization of the solid phase reactant. A solid phase which is pre-coated with an analyte-specific solid phase reactant is particularly preferably afterwards blocked with an analyte-unspecific biomolecule.

Good results were obtained by using solid phases which have "defined test" areas i.e. defined zones that are coated with a solid phase reactant which are spatially separated from other test areas by inert zones. Solid phases that are coated over the whole area with an analyte-unspecific pre-coating e.g. with streptavidin and which contain at least one spatially limited test area immobilized on the one analyte-specific solid phase reactant are particularly preferred. The defined test areas preferably have a diameter of 10 μm to 10 mm. Miniaturized test areas are particularly preferred with a diameter of 10 μm to 2 mm. Furthermore solid phases are preferred with several test areas which can contain different analyte-specific solid phase reactants and are also referred to as array systems (cf. e.g. US Patent 5,432,099; 5,516,635 and 5,126,276). These array systems enable several analyte determinations to be carried out simultaneously on one sample.

The solid phase in the method according to the invention comprises an arbitrary support, non-porous supports such as supports with a plastic, glass, metal or metal oxide surface being preferred. Porous supports such as test strips are also suitable.

An analyte-specific solid phase reactant i.e. a biomolecule which can specifically interact with an analyte to be determined or, in the case of competitive test formats, with an analogue of an analyte to be determined, is immobilized on the solid phase. Examples of analyte-specific solid phase reactants are antibodies, antigens, peptides, haptens, nucleic acids, nucleic acid analogues, glycoproteins, saccharides, lipoproteins and other biomolecules.

The solid phase reactant can be immobilized by known methods e.g. by direct adsorptive binding, by covalent coupling or preferably by coupling via high affinity binding pairs. For this the solid phase is firstly coated with a first partner of a high affinity binding pair and on this a conjugate of the solid phase reactant with the second partner of the binding pair is immobilized. Examples of suitable high affinity binding pairs are streptavidin or avidin/biotin or a biotin derivative (for example desthiobiotin, iminobiotin, aminobiotin or another substance capable of binding with high affinity to streptavidin or avidin), antibody/hapten (for example digoxigenin, fluorescein etc.), antibody/antigen (for example peptide or polypeptide), lectin/sugar and receptor/ligand (for example hormone receptor/hormone). Streptavidin or avidin/biotin are particularly preferably used as the high affinity binding pair.

The method according to the invention preferably comprises blocking unspecific binding sites on the solid phase that is already coated with the analyte-specific solid phase reactant by incubation with an alkylene oxide-modified binding molecule which acts as a blocking substance. The duration and temperature of the incubation can be varied within wide ranges e.g.

incubation temperatures of 4°C to 40°C and incubation times of 1 min to 1 h.

Suitable blocking substances are analyte-unspecific or inert biomolecules which are capable of binding to the solid phase and do not interfere with the detection method, for example proteins such as albumins, unspecific antibodies or fragments thereof, or polysaccharides such as dextrins etc.. The blocking substance can be bound to the solid phase by means of adsorptive or covalent interactions. However, binding by means of high affinity binding pairs is preferred. In particular in the case of a solid phase which contains the solid phase reactants immobilized by means of a high affinity binding pair, a blocking substance is preferably used which comprises the second partner of the binding pair e.g. a biotinylated protein which contains one or several polyalkylene oxide residues. Alternatively the use of blocking substances is also preferred in which one or several polyalkylene oxide residues are directly coupled to the second partner of the binding pair.

Preferred blocking substances are conjugates of the general structural formulae (Ia) or (Ib):

B

$(AO)_n$

$$P_r[-\cancel{(AO_n)}T]_m \quad (Ia)$$

$(AO)_n$

$$P_r-I-[-\cancel{(AO_n)}T]_m \quad (Ib)$$

B

in which

P is a partner of a high affinity binding pair,

I is a biomolecule,

r is a number from 1 to 10,

AO is a (C_2-C_3) alkylene oxide group,

n is a number from 5 to 500

T is an end group preferably selected from OH, C_1-C_4 alkoxy and acyl and

m is a number from 1 to 10.

P is preferably a hapten, biotin or a biotin derivative. P is particularly preferably biotin or a biotin derivative. I is preferably a polypeptide or a saccharide. In conjugates of formula (Ia) r is preferably 1.

AO can be a (C_2-C_3) alkylene oxide group i.e. an ethylene oxide or/and a propylene oxide group. AO is preferably an ethylene oxide group but combinations of ethylene oxide and propylene oxide groups are also suitable. n is preferably a number from 10 to 250 and particularly preferably 20 to 200.

T is an end group (including the terminal O atom of the polyoxyalkylene units), which is compatible with further test and sample components, i.e. which does not significantly enter undesired reactions. Preferably T is a hydroxy group, C_1-C_4 alkylether group, particularly methoxy, or a C_1-C_4 acyl group, e.g. an acetyl group. In conjugates of the structural formula (Ia) m is preferably 1.

The conjugates according to structural formula (Ia) and (Ib) are preferably used as blocking reagents in detection methods. After immobilization on a solid phase, they are preferably no longer able to bind with high affinity via the component P to dissolved biomolecules in the sample or in the test reagent.

A further subject matter of the invention is a solid phase with a coating which contains one or several conjugates (Ia) or/and (Ib) and preferably an analyte-specific solid phase reactant. The conjugates according to the invention can be used to reduce unspecific binding to a solid phase in a method for the detection

of an analyte for example in an immunological or a nucleic acid hybridization method. A further subject matter of the first aspect of the present invention is a reagent kit for the detection of an analyte which contains a conjugate according to the invention or a solid phase according to the invention in addition to other test components.

In a particularly preferred embodiment biotin-polyethylene glycol compounds are used which are PEG chains which have been functionalized with a biotin residue at one chain end. The other chain end preferably carries a hydroxyl or a methoxy group. The biotin-PEG conjugates are applied to a steptavidin solid phase after or at the same time as a biotinylated analyte-specific solid phase reactant e.g. an antibody. The conjugate binds to the free biotin binding sites of the streptavidin solid phase that are still accessible. The non-bound biotin-PEG conjugate can be removed by washing. The resulting solid phase can be dried in this state without impairing the function. The unspecific binding of a surface treated with a conjugate according to the invention is greatly reduced compared to an untreated surface or compared to a surface treated with a non-alkylene-oxide-modified blocking substance. A further advantage is that the solid phase according to the invention can also be treated with the blocking conjugate and thus provided with the desired properties after application of the solid phase reactant. In solid phases which have defined test areas and a continuous pre-coating, a considerable reduction of unspecific binding is found within the test areas as well as outside these test areas (e.g. empty streptavidin solid phase). The ability of the solid phase to bind the analyte remains surprisingly uninfluenced.

A second aspect of the present invention is a method for the detection of an analyte in a sample comprising the steps:

(a) preparing a solid phase on which a solid phase reactant is immobilized using a modified solid phase reactant which is coupled to a poly(C_2-C_3)-alkylene oxide,
(b) incubating the sample with the solid phase and a test reagent and
(c) detecting the presence or/and the amount of the analyte in the sample.

According to this second aspect of the invention a polyalkylene oxide modified solid phase reactant is immobilized on the solid phase. On the one hand, the modified solid phase reactant can be a universal solid phase reactant i.e. a reactant which cannot react specifically with the analyte but rather with a further solid phase reactant that is immobilized covalently, adsorptively or via a high affinity binding pair on the solid phase. Examples of universal solid phase reactants are for example streptavidin or anti-hapten antibodies which can react with a biotinylated or hapten-conjugated analyte-specific additional solid phase reactant. On the other hand or additionally the analyte-specific solid phase reactant can also be a polyalkylene oxide-modified solid phase reactant.

A universal modified solid phase reactant can for example be a partner of a high affinity binding pair or a conjugate of an analyte-unspecific biomolecule with a partner of a high affinity binding pair. Examples of universal solid phase reactants which are themselves the partner of a high affinity binding pair are polypeptides such as streptavidin, avidin, hapten-specific

antibodies, lectins and polymeric conjugates thereof. On the other hand, it is also possible to use a conjugate of an analyte-unspecific biomolecule with a partner of a high affinity binding pair as a universal solid phase reactant, for example an inert polypeptide or polysaccharide coupled to biotin, biotin derivatives, haptens or sugars.

Even when using an analyte-specific modified solid phase reactant it is preferable that this is a conjugate with a partner of a high affinity binding pair. Examples of such analyte-specific modified solid phase receptors are analyte-specific antibodies, antigens, nucleic acids, nucleic acid analogues and lectins.

In one embodiment of the second aspect of the present invention conjugates of the general structural formula (II) are used:

$$F[-\overset{(AO)_n}{\cancel{(AO_n)}}T]_m \qquad \text{(II)}$$

in which

F is a polypeptide selected from lectins, streptavidin, avidin and antibodies,
r is a number from 1 to 10,
AO is a C_2-C_3-alkylene oxide group,
n is a number from 5 to 500,
T is an end group preferably selected from OH, C_1-C_4 alkoxy and C_1-C_4 acyl and
m is a number from 1 to 10

and in which the conjugates preferably have at least one binding site which, after immobilization of the conjugates, on a solid phase, can still bind with high affinity to a soluble reactant.

In a further preferred embodiment, conjugates of the general structural formula (III) are used:

$$P_{r'} \cdot F_r[-(AO)_n-T]_m \qquad \text{(III)}$$

in which
P' is a partner of a high affinity binding pair,
r' is a number from 1 to 10,
F is a biomolecule,
r is a number from 1 to 10 and
AO, n, T and m are defined as for conjugates of structural formula (II).

Conjugates of the structural formulae (II) and (III) are preferably used as a universal or analyte-specific solid phase reactant in detection methods.

The second aspect of the present invention also concerns a solid phase with a coating which contains conjugates of the general structural formula (II) or/and (III). The conjugates can be used to reduce unspecific binding to a solid phase in a method for the determination of an analyte for example in an immunological method or in a nucleic acid hybridization method. The second aspect of the invention additionally concerns a reagent kit for the detection of an analyte which contains a conjugate of the general structural formula (II) or (III) or a solid phase coated with such a conjugate in addition to other test components.

A third aspect of the present invention concerns a method for the detection of an analyte in a sample comprising the steps:

(a) preparing a solid phase on which an analyte-

specific solid phase reactant is immobilized,

(b) incubating the sample with the solid phase and a test reagent in which the test reagent contains an analyte-specific modified soluble reactant which is coupled to a poly(C_2-C_3)-alkylene oxide and

(c) detecting the presence or/and the amount of the analyte in the sample.

In this aspect of the invention a modified soluble analyte-specific reactant is used i.e. a biomolecule which can specifically bind to an analyte or/and analyte analogue to be determined. The modified soluble reactant can be directly labelled i.e. carry a labelling group e.g. an enzyme, fluorescent or electrochemiluminescent labelling group. On the other hand the soluble reactant can also be indirectly labelled i.e. it carries a group that can react with a detectable labelling group e.g. a hapten which can in turn react with a labelled anti-hapten antibody.

The modified soluble reactant is preferably selected from antibodies, antigens, nucleic acids, nucleic acid analogues and lectins.

According to this third aspect of the present invention conjugates of the general structural formula (IV) are preferably used:

$$M_s\text{-}F''\text{-}[-(AO)_nT]_m \qquad \text{(IV)}$$

in which

M is a labelling group or a group that can react with a labelling group,

s is a number from 1 to 10,

F" is a soluble biomolecule in particular selected from antibodies, antigens, nucleic acids, nucleic acid analogues and lectins and
AO, n, T and m are defined as for conjugates of the structural formula (II).

These conjugates can be used to reduced unspecific binding to a solid phase in a method for the determination of an analyte, in particular in an immunological determination method, a nucleic acid hybridization method or a sugar-lectin determination method. Furthermore this third aspect of the present invention concerns a reagent kit for the detection of an analyte which contains a conjugate of the general formula (IV) in addition to other test components.

A fourth aspect of the present invention concerns a method for reducing unspecific binding to a solid phase in a method for the detection of an analyte in a sample characterized in that at least one reagent is used which contains a substance coupled to a poly(C_2-C_3)-alkylene oxide.

The substance coupled to a poly(C_2-C_3)-alkylene oxide is preferably selected from

(i) blocking substances,
(ii) universal solid phase reactants
(iii) analyte-specific solid phase reactants and
(iv) soluble reactants.

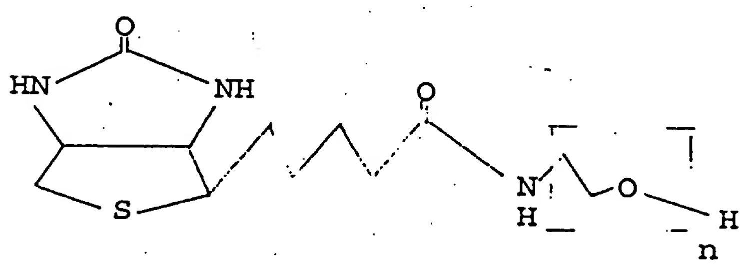
It is preferable to use more than one alkylene oxide modified class of substances in the method.

One subject matter of this fourth aspect of the invention is a reagent kit for the detection of an analyte comprising at least one reagent which contains a substance coupled to a poly(C_2-C_3)-alkylene oxide which is preferably selected from one of the previously mentioned substance classes.

The invention is further elucidated by the following examples.

Examples

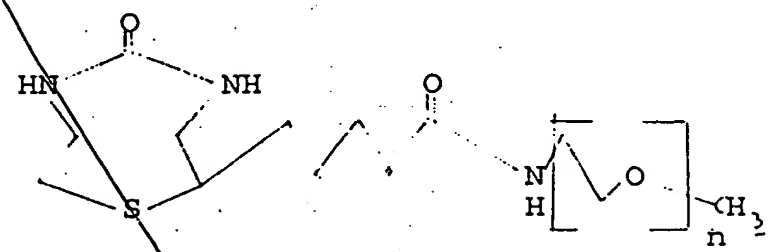
1. Synthesis of a biotin-polyethylene glycol(PEG) conjugate (MW3499)



n~77

550 mg 1-amino-PEG (Shearwater Polymers Co.) was dissolved in 10 ml dioxane. 60 mg triethylamine was added to this solution and subsequently 100 mg biotin-OSu-ester (Boehringer Mannheim) was added. The mixture was stirred for 2.5 hours at room temperature. Afterwards the product was purified by column chromatography. The yield was 30%.

2. Synthesis of a biotin-methoxypolyethylene glycol conjugate (MW 5000)



n~114

150 mg Aminomethoxy-PEG (Sigma Co.) was dissolved in 100 ml dioxane and subsequently 2 g biotin-OSu ester dissolved in 60 ml DMF was added. After addition of 40 mg triethylamine it was stirred for 3 hours at room temperature and then for a further 3 hours at 70°C. The solvent was subsequently removed and the product was purified by column chromatography. The yield was 57 %.

3. Preparation of biotin-PEG solid phase

Biotinylated antibodies to TSH were applied in the form of areas with a diameter of ca. 0.1 mm by means of a microdosing technique to a streptavidin solid phase (p-styrene coated with streptavidin bound to thermally polymerized bovine serum albumin (BSA)). After application of the antibody zones this solid phase was retreated with phosphate buffer pH 7.5 containing 50 µg/ml Bi-PEG. It was rewashed after 2 minutes incubation and dried.

4. Investigation of unspecific binding to the Bi-PEG coated solid phase

The solid phase prepared by the method described in

example 3 was evaluated with the following system: After incubation with analyte-free sample material (p24-free human serum or Enzymun®-TSH 0 standard) and a subsequent wash step, the solid phases that had been incubated with human serum were incubated with digoxygenilated detection reagent (p24-Dig conjugate and with anti-human IgG-antibody-Dig conjugate). The digoxygenilated reagents are not specific for the anti-TSH antibodies i.e. they do not contain an analyte but represent a marker for the level of unspecific binding. After a wash step, the unspecific binding was determined by a fluorescent dyed latex that was labelled with anti-Dig antibodies. The signals obtained by fluorescent microscopic techniques were quantified by optical image evaluation and stated as counts/sec. The fluorescence intensity within the test areas (containing biotinylated TSH antibodies) and outside the test areas (background without antibody coating) were measured.

Table 1: Results in the test area (containing TSH antibodies) in counts/sec

Solid phase	Detection reagent		
	TSH 0 standard	p24-Dig	<h-IgG>-Dig
without Bi-PEG	65	1897	1612
with Bi-PEG	31	740	1231

Table 2: Results in the background (streptavidin solid phase) in counts/sec

Solid phase	Detection reagent		
	TSH 0 standard	p24-Dig	<h-IgG>-Dig
without Bi-PEG	51	766	654
with Bi-PEG	17	136	140

In all cases the addition of Bi-PEG led to a considerable reduction of unspecific binding on the solid phase.

5. Synthesis of a streptavidin-polyethylene glycol conjugate

Streptavidin and PEG-OSu were dissolved in phosphate buffer and added together in the stoichiometry desired in each case, preferably 1:1 to 1:5. After 2 h reaction at room temperature (?), the reaction mixture was dialysed against phosphate buffer containing 0.05 % sodium azide and stored at 4°C.

6. Preparation of a universal streptavidin-PEG solid phase

A reaction vessel was filled with a solution which contained biotinylated carrier protein (BSA-biotin or thermo-BSA-biotin) at a concentration of 100 µg/ml and incubated for 5 min at room temperature. Then the solution was aspirated and the coated reaction vessel was rinsed with phosphate buffer and again aspirated.

Subsequently streptavidin-PEG was added at a concentration of 50 µg/ml in phosphate buffer containing 1 % BSA and incubated for 15 min. Afterwards the solution was aspirated and washed by adding phosphate buffer containing 1 % BSA and 2 % sucrose. After again aspirating and drying, the solid phase was stored at 4°C in air-tight packaging.

7. Preparation of specific streptavidin-PEG solid phase

A reaction vessel was filled with a solution which contained biotinylated carrier protein at a concentration of 100 µg/ml and incubated for 5 min. The solution was aspirated, rinsed with phosphate buffer and again aspirated. Then streptavidin-PEG (50 µg/ml) in phosphate buffer containing 1 % BSA was added and it was incubated for 15 min. The solution was aspirated, rinsed with phosphate buffer and again aspirated.

Then a biotinylated antibody e.g. a monoclonal anti-TSH-antibody Fab'$_2$ fragment (5 µg/ml) was added and incubated for 15 min. The solution was aspirated and rinsed by adding phosphate buffer containing 1 % BSA and 2 % sucrose and again aspirated. After drying, the solid phase was stored at 4°C in air-tight packaging.

8.1 Evaluation of streptavidin-PEG solid phases

A reaction vessel containing the solid phase from example 6 or 7 was incubated for 20 min at room temperature with a prediluted analyte-free sample (horse serum diluted 1:1 with loading buffer 50 mM Tris/HCl pH 7.5, 0.5 % BSA, 0.05 % Tween 20, 0.9 % NaCl). After washing, it was incubated for 20 min in the presence of

signal antibody (1 μg/ml monoclonal anti-TSH-antibody IgG-digoxigenin conjugate in loading buffer) and washed again.

After addition of the detection reagent (0.01 % solution of fluoro beads coated with monoclonal anti-digoxigenin antibody IgG) it was incubated for 20 min, washed and the fluorescence signal was measured.

Table 3: Fluorescence blank values (arbitrary units) on various solid phases

	unspecific solid phase	specific solid phase
SA underivatized	199	373
SA-PEG (1:1)	114	114
SA-PEG (1:5)	(100)	(100)

8.2 Unspecific binding of buffer components

A reaction vessel containing the solid phase from example 6 or 7 was filled with a horse serum sample as described in example 8.2 and washed. Then 0.2 μg/ml p24-digoxigenin in loading buffer was added, incubated for 20 min and it was washed. Then the detection reagent (cf. 8.1) was added, incubated again for 20 min, washed and the fluorescence signal was measured. The results are shown in table. 4.

Table 4: Unspecific binding of p24-digoxigenin (arbitrary units) on various solid phases

	unspecific solid phase	specific solid phase
SA underivatized	691	(>1500)
SA-PEG (1:1)	260	660
SA-PEG (1:5)	124	365

8.3 Unspecific binding of human IgG antibodies

A reaction vessel containing the solid phases prepared in examples 6 and 7 was filled with a sample as described in example 8.1 and washed. The sample was human serum, diluted 1:19 with loading buffer.

Then 1.0 µg/ml monoclonal anti-human IgG-antibody-digoxigenin conjugate in loading buffer was added and it was washed. Subsequently the detection reagent was added, incubated for 20 min, washed again and the fluorescence signal was measured. The results are shown in the following table 5.

Table 5: Unspecific binding of human antibodies (arbitrary units) on various solid phases

	unspecific solid phase	specific solid phase
SA underivatized	2549	(>3000)
SA-PEG (1:1)	944	1749
SA-PEG (1:5)	515	834

9. Preparation of antibody-PEG conjugates

PEG-antibody conjugates were prepared as described in example 5 except that a biotinylated antibody was used instead of streptavidin.

10. Preparation of solid phases coated with PEG-antibody conjugates

A reaction vessel was incubated for 5 min with a solution which contained 100 µg/ml biotinylated carrier protein (BSA-biotin or tBSA-biotin). Then the solution was aspirated, rinsed with phosphate buffer and aspirated again.

Subsequently 50 µg/ml streptavidin in phosphate buffer containing 1 % BSA was added and it was incubated for 15 min. This solution was aspirated, rinsed with phosphate buffer and again aspirated. Afterwards 5 µg/ml biotinylated IgG antibody e.g. a monoclonal anti-TSH-Fab'$_2$ antibody fragment was added and it was incubated for 15 min. The solution was aspirated and a rinsing step with phosphate buffer, 1 % BSA, 2 % sucrose was carried out. After again aspirating, the reaction vessel was dried and stored at 4°C in air-tight packaging.

11. Evaluation

11.1 Blank value

The blank value of the solid phase prepared in example 10 was determined as described in example 8.1. The results are shown in table 6.

Table 6: Fluorescence blank values (arbitrary units) on various solid phases

	Signals (arbitrary units)
AB underivatized	270
AB-PEG (1:1)	94
AB-PEG (1:5)	57

11.2 Unspecific binding of buffer components

The unspecific binding of buffer components to the solid phase prepared in example 10 was determined as described in example 8.2. The results are shown in table 7.

Table 7: Unspecific binding of p24-digoxigenin (arbitrary units) on various solid phases

	Specific solid phase
AB underivatized	26658
AB-PEG (1:1)	23519
AB-PEG (1:5)	7998

11.3 Determination of the unspecific binding of human antibodies (IgG)

The determination of the unspecific binding of human IgG antibodies to the solid phase prepared in example 10 was carried out as described in example 8.3. The results are

shown in Table 8.

Table 8: Unspecific binding of human antibodies (arbitrary units) on various solid phases

	Specific solid phase
AB underivatized	11379
AB-PEG (1:1)	10475
AB-PEG (1:5)	4446

Example 12 Procedure for a <HIV I> test and test results with negative samples

An antigen which represents the gp41 of the HIV I virus was applied to a test area of ca. 100 μm diameter on a polystyrene support. 30 μl sample that had been prediluted with sample buffer was pipetted onto the test area and incubated for 20 minutes at room temperature while shaking. After aspirating the sample and washing the test zone with wash buffer, 30 μl reagent solution containing a Dig-labelled gp41 which represents the HIV I antigen was added by pipette and it was again incubated for 20 minutes at room temperature while shaking. After aspirating the reagent solution and washing the test zone with wash buffer, 30 μl detection reagent was pipetted onto the test zone. Fluorescence-dyed latex particles of 100 nm size serve as the detection reagent which are covalently coated with an anti-Dig antibody.

This detection reagent was in turn incubated for 20 minutes at room temperature while shaking, subsequently aspirated, washed and sucked dry. The test zone was irradiated with a HeNe laser at 633 nm wavelength and the fluorescence at 670 nm wavelength was measured with a CCD camera.

The following test-specific reagents were used:

solid phase antigen:	polyhapten composed of gp41 peptide
detection antigen:	polyhapten composed of gp41-peptide, labelled with Dig.

The following measured values (counts) were measured:

Sample	background* [counts]	signal test zone [counts]	Signal test zone background	cut-off index**
negative control	148	148	0	0.0
positive sample 1	172	26435	26257	88.1
positive sample 2	172	22908	22376	76.8
negative sample 1	101	101	0	0.0
negative sample 2	103	103	0	0.0
negative sample 3	93	93	0	0.0
negative sample 4	98	98	0	0.0
negative sample 5 (S441)	86	4401	4315	14.6
negative sample 6 (S480)	137	2690	2553	8.6
negative sample 7 (S486)	107	3833	3726	12.6
negative sample 8 (S520)	116	4331	4215	14.2

* the background corresponds to the signal adjoining the test zones

** the cut-off index = $\text{signal}_{\text{sample}} - \text{signal}_{\text{background}} / 2 \times \text{signal}_{\text{negative control}}$

cut-off index < 1 = negative

The above table represents an extract from a specificity study. Ca. 240 <HIV I> negative samples were measured in this study. Most of the samples (e.g. negative samples 1-4) exhibited no reaction on the test zones and were therefore unequivocally negative. However, four samples (negative samples 5-8) were found which exhibited a strong unspecific reaction on the test zones and were thus detected as false positive.

Example 13 Improvement of the specificity by a PEG-derivatized solid phase antigen

In this experiment a <HIV I> test was carried out analogously to example 12. In contrast thereto, an identical antigen which was derivatized with PEG 500 in a stoichiometry ratio of 1:1 was additionally applied to the identical test support next to the HIV I antigen.

The following measured values were obtained:

Sample	background* [counts]	polyhapten-gp41-peptide		polyhapten-gp41-peptide-PEG	
		Counts**	COI***	Counts**	COI***
negative control	52	0	0.0	31	0.3
positive control	63	286	2.0	8383	80
positive sample 1	212	11752	111	6227	57.8
positive sample 2	84	1632	14.9	3762	35.3
S441	50	1061	9.7	79	0.3
S480	53	871	7.9	102	0.5
S486	44	1041	9.6	98	0.5
S520	44	1260	11.7	84	0.4

* the background corresponds to the signal adjoining the test zones

** $\text{signal}_{\text{sample}}\text{-signal}_{\text{background}}$
*** COI = cut-off index = $\text{signal}_{\text{sample}}\text{-signal}_{\text{background}}/$
$2 \times \text{signal}_{\text{negative control}}$
cut-off index < 1 0 negative

This result shows that the unspecific binding of the interfering samples in the <HIV I> test zone is substantially reduced by using the new PEG-derivatized antigen so that all 4 interfering samples are negative. Surprisingly the PEG-derivatization was even able to lead to a strong increase of the signal of positive samples (see positive control and positive sample 1).

14. Detection of HBs-antigen

A monoclonal antibody to HBs antigen was applied to a test area of ca. 100 µm diameter on a polystyrene support. The same antibody in the form of a PEG conjugate (preparation example 9) was applied to another test area. 30 µl sample prediluted with sample buffer was pipetted onto the test area and incubated for 20 min at room temperature while shaking. After aspirating the sample and washing the test area with wash buffer, 30 µl reagent solution containing digoxigenin-labelled anti-HBsAg antibody was added by pipette and it was again incubated for 20 min at room temperature while shaking. After aspirating the solution and washing the test area with wash buffer, 30 µl detection reagent (example 8.3) was pipetted onto the test area.

The detection was carried out as described in example 8.1.

The following were examined: a positive standard, a

negative standard as well as five negative sera which contain no HBsAg but nevertheless yield significant positive signals in the test which were due to analyte-unspecific interactions with the solid phase. Results of these experiments are listed in table 9. It can be clearly seen that the unspecific binding of PEG-derivatized antibodies is very much lower than that of untreated antibodies.

Table 9

	Measured signal	
Sample	**MAB<HBs>**	**MAB<HBs>PEG**
pos. standard	1080	960
neg. standard	1.3	1.7
negative serum 1	16	3.4
negative serum 2	28	12
negative serum 3	15	1.3
negative serum 4	11.5	2
negative serum 5	18	9